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Visualization of Diagnostic Heteroduplex DNAs from Cystic Fibrosis Deletion Heterozygotes Provides an Estimate of the Kinking of DNA by Bulged Bases*

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Previous studies (Hsieh, C.-H., and Griffith, J. D. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 4833–4837) of DNAs containing extra or bulged bases on one of the two strands of a duplex showed that they exhibit slower electrophoretic mobility than non-bulged DNAs, indicating that bulges create stiff kinks in the DNA. Here we paired a 97-base single-stranded DNA from the intact cystic fibrosis (CF) gene with a complementary 94-base strand containing a central 3-base deletion (ΔF_{508}), common to many CF patients. This produced a 94-base pair DNA with a central 3-base bulge. Visualization of these DNAs by electron microscopy showed that twice as many bulge-containing DNAs had a central kink as compared with the non-bulged controls. Examination of the distribution of kinking angles showed that the bulged population contained 5–7-fold more molecules with a central kink of 80 ± 10° than did the control molecules. When the 3-base bulge was replaced by a 3-base gap, the resulting duplex DNA showed central kinks with a somewhat lower frequency but greater range of kinking angles.

The discovery of human diseases produced by small deletions in the chromosomal DNA has focused interest on understanding the nature of these lesions and the events that lead to their generation. One likely intermediate in the pathway through which small deletions are fixed into the chromosome are extra base bulges produced during DNA replication and/or recombination. NMR studies indicate that bulged bases can either stack into the helix or loop out depending on the number of bases in the bulge, the composition of the bulge, and other factors (1–4). Gel electrophoretic studies carried out in this and the laboratories of Crothers and Lilley (5–8) have demonstrated that bulges of 1–5 bases generate DNAs with greatly reduced electrophoretic mobilities in polyacrylamide gels suggesting that bulged bases kink DNA.

The demonstration that bulged bases produce electrophoretic retardations has provided a means of screening for small chromosomal deletions or insertions. For example, nearly 70% of the carriers of CF[1] in the western Caucasian population possess a single 3-base deletion (ΔF_{508}) in the CF gene on chromosome 7 (9). Several groups have used PCR to amplify small regions of DNA (100 bp) around codon 508 of the CF gene (10–12); if the DNA is from a carrier of the disease and thus a heterozygote, amplification produces four single-stranded DNA fragments, two that contain the deletion and two that do not. Upon annealing the four DNA strands, two homoduplexes and two heteroduplexes (one containing a CTT bulge and the other containing an AAG bulge) are produced, and the latter species can be detected by their retarded electrophoretic mobility. This approach has also been used to screen for a 4-base insertion mutation in Tay-Sachs disease (13, 14).

For these genetic screens to be the most useful and to understand how bulged bases in DNA lead to mutations, a detailed elucidation of the structure and dynamics of bulges is needed. As a step in this direction, we recently examined the dependence of electrophoretic retardations due to single-base bulges both on the base comprising the bulge and its neighboring base pairs (8). Using ultraviolet absorption techniques, Morden and colleagues (15) studied the effect of base composition and flanking base pairs on the thermostability of bulge-containing DNA and obtained results similar to our gel electrophoresis study. Although these studies have added to our knowledge of bulged bases, a major gap remains. There has been no direct measurement of the kinking angle produced in a DNA by a bulged base, nor is there any information about the distribution of kinking angles within the population of bulged DNAs. In our initial study (6) and that of Rice and Crothers (7), the electrophoretic retardations of bulged DNA were compared with that of kinetoplast DNAs containing phased tracts of adenines to provide a rudimentary estimate of the kinking angle due to one or more bulged bases. However, as we recently observed by EM (16), the kinetoplast DNAs do not appear as a single bent species with each molecule bent to the same degree, but rather a population in rapid transition between states ranging from not bent to highly bent with some average state that is detected by gel electrophoresis. If this is so, it compromises the use of the kinetoplast DNAs as a measure of the kinking or bending of DNA due to factors that might produce different distributions of straight and kinked molecules. For this reason it seemed of value to directly visualize bulge-induced kinks in DNA by EM, to demonstrate conclusively that bulges kink DNA and to provide information on the distribution of kinking angles in the population of bulged DNAs.

In this study DNA was obtained from a CF carrier and the CF homoduplex, and bulge-containing heteroduplex molecules were prepared as described above. These DNAs were

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[1] The abbreviations used are: CF, cystic fibrosis; PCR, polymerase chain reaction; bp, base pair(s); EM, electron microscopy.

also directly synthesized, and both sets of DNAs were examined by EM for the presence of visible kinks. Twice as many bulge-containing DNAs were seen to have a central kink compared with the non-bulged controls. Inspection of the angular distribution of kinks showed that the presence of a single 3-base bulge produced a much greater number of molecules containing a kink of 80 ± 10° than was found in the non-bulged DNAs. Gel electrophoresis of the synthetic CF DNAs confirmed that the CTT bulge kinks DNA less than an AAG bulge.

EXPERIMENTAL PROCEDURES

Preparation of PCR-amplified CF DNA—Genomic DNA was obtained from peripheral blood of a ΔF_{508} deletion heterozygote individual, and exon 10 of the CF gene was amplified by PCR (17) with primers bracketing nucleotides 1611–1707 (the first nucleotide position corresponds to the first base in the 5′ extension clone PA3–5 (17)) (Fig. 1*A*). The PCR-amplified products were annealed and then electrophoresed on 5% polyacrylamide gels (38:2 acrylamide: bisacrylamide ratio) in 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0, buffer at 250 V for 14 h at 20 °C. Two sets of bands corresponding to the two homoduplex and two heteroduplex species were detected after staining the gel with ethidium bromide (0.5 μg/ml in electrophoresis buffer). The DNA from each band was extracted from the gel.

Preparation of Synthetic CF DNA—Oligonucleotides with the sequence between codons 496 and 519 (Fig. 1*A*, *arrows*) were synthesized with *Bam*HI and *Eco*RI elements added at 5′ and 3′ ends, respectively. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer and purified by polyacrylamide gel electrophoresis. The complementary oligonucleotides were hybridized, and the molecule was made fully duplex using the large fragment of DNA polymerase I to produce the individual homoduplex (85 or 82 bp) and heteroduplex (containing CTT or AAG bulges) DNAs. A DNA designed to be more flexible than the bulged DNA was produced by annealing one 85-base oligonucleotide with two 41-base oligonucleotides. The sequence of this DNA is the same as that of the CTT-bulged heteroduplex, except that it contains a 3-base gap opposite the CTT of the bulge (Fig. 1*B*).

Gel Electrophoresis—DNA duplexes were electrophoresed on 15% polyacrylamide gels (30:1 acrylamide:bisacrylamide ratio) in 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0, buffer at room temperature. Samples were visualized by staining with ethidium bromide.

Electron Microscopy—DNA samples were mixed with a buffer containing 0.4 mM spermidine and briefly adsorbed to a thin carbon film, washed, dried, and rotary shadowcast with tungsten at 1.3×10^{-5} Pa in an oil-free cryopumped system (18). Micrographs were taken on a Philips EM 400TLG instrument. Molecule lengths were measured by projecting images of the DNA on the micrographs onto a Summagraphics digitizing tablet coupled to a CompuAdd computer programmed with software developed in this laboratory. Kinking angles were measured with a protractor.

RESULTS

Genomic DNA was obtained from a ΔF_{508} deletion heterozygote individual and exon 10 of the CF gene was amplified by PCR (see "Experimental Procedures" and Fig. 1*A*). Electrophoresis of the annealed fragments on a polyacrylamide gel (not shown) revealed two doublet bands, one doublet containing the two homoduplexes and the other doublet containing the two heteroduplexes (one with a CTT bulge and the other with an AAG bulge). The DNA from each doublet was extracted from the gel as a heteroduplex pool and a homoduplex pool. To provide individual heteroduplex species and to exclude any possible PCR amplification of undesired species, oligonucleotides (Fig. 1*A*) were synthesized to produce the individual homoduplexes and heteroduplexes. For comparison, a DNA designed to be more flexible than the bulged DNA was synthesized consisting of the same sequence as the CTT-bulged DNA, except that a 3-base gap replaced the CTT bulge (Fig. 1*B*). Gel electrophoresis (Fig. 1*C*) showed that the heteroduplex containing the AAG bulge migrated

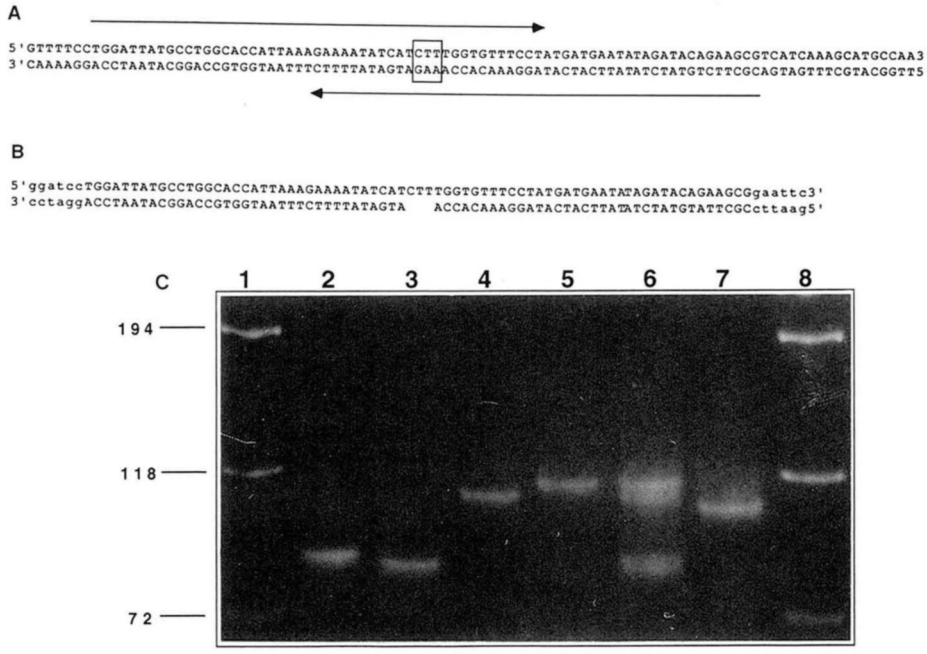

FIG. 1. *A*, sequence of a segment of exon 10 of the CF gene. A 97-bp segment of exon 10 beginning with codon 494 was amplified by PCR. The 3-base deletion at codon 508 is shown in the *box*. Oligonucleotides with the sequence between codons 496 and 519 (*arrows*) were synthesized to produce the individual homoduplex (85 or 82 bp) and heteroduplex (containing CTT or AAG bulges) DNAs. The bulges are 45 and 50% from the 5′ end of the PCR-amplified DNAs and synthetic DNAs, respectively. *B*, sequence of the CF DNA containing a 3-base gap. *C*, gel electrophoresis of bulge-containing CF DNAs. *Lanes 1* and *8*, *Hae*III-cleaved φX174 DNA fragments; *lane 2*, the 85-bp CF segment DNA; *lane 3*, the 82-bp DNA containing the 3-base deletion in both strands; *lane 4*, the DNA containing the CTT bulge; *lane 5*, the DNA containing the AAG bulge; *lane 6*, all four DNAs; *lane 7*, the DNA containing the 3-base gap. The sizes of φX174 fragments are indicated.

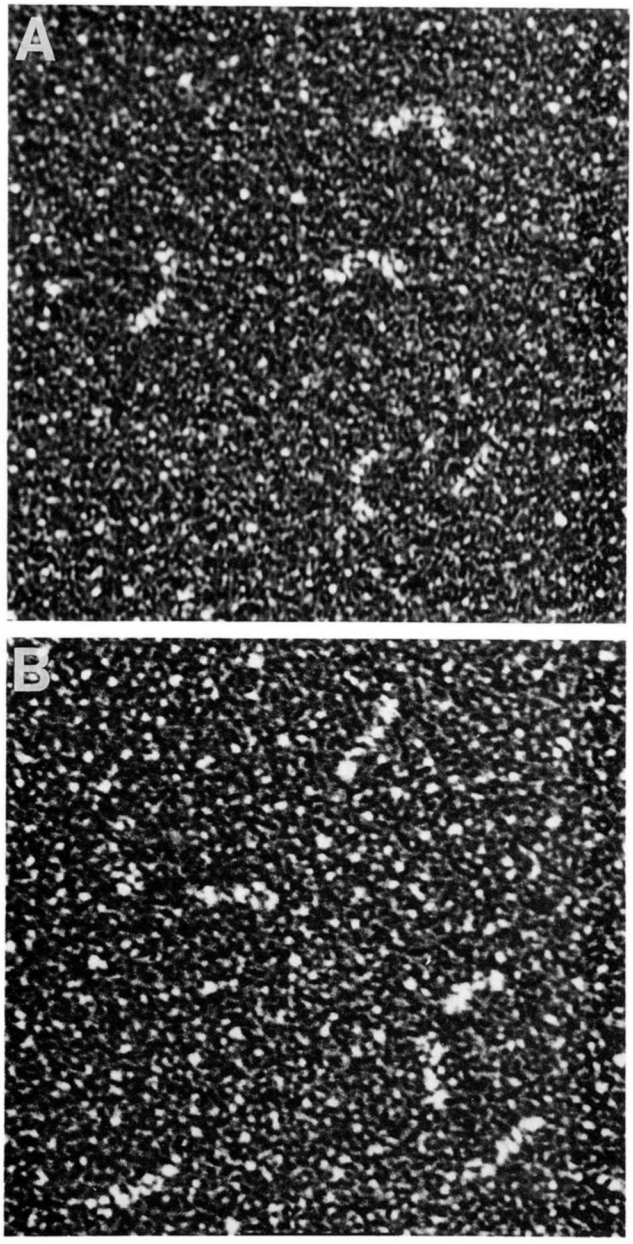

FIG. 2. **Visualization of CF DNAs.** CF DNAs containing an AAG bulge at its center (*A*) or no bulge (*B*) were visualized by transmission EM as described under "Experimental Procedures" including rotary shadowcasting with tungsten. Micrographs were taken on a Philips EM 400TL. Shown in reverse contrast. *Bar* equals 500 Å.

more slowly than the CTT-bulged DNA, as predicted (8); the electrophoretic mobility of the 3-base gapped DNA was slightly faster than that of the CTT-bulged DNA.

These DNAs were prepared for EM (see "Experimental Procedures"). In fields containing many molecules (Fig. 2), the number that were either straight or contained a sharp central kink of approximately 30° or greater were scored by eye. Each molecule with such a kink was photographed, and the position of the kink relative to the ends of the DNA was measured. For molecules in which the kink was within 10% of the center, the angle of the kink was determined. For this study over 2500 molecules were scored.

EM analysis of the pooled heteroduplexes and the pooled homoduplexes from the PCR amplification showed that twice as many bulge-containing DNAs had a central kink of 30° or more compared with the non-bulged homoduplex DNAs (Table I). The angular distribution of kinks (Fig. 3) showed that whereas most of the molecules scored as being kinked in the non-bulged DNAs were kinked by 30–60°, very few had kinks of 70–90° (note that a cut-off of angle less than 30° was imposed by eye and a kinking angle of 0° represents a perfectly straight molecule). In contrast, in the bulge-containing DNAs, 5–7-fold more were kinked by 70–90° (Table I, Fig. 3).

TABLE I

Electron microscopic analysis of PCR-amplified and scored synthetic cystic fibrosis DNA (2584 molecules scored)

DNAs amplified by PCR (17) from the DNA of a CF carrier were annealed to produce homoduplexes and heteroduplexes and fractionated on polyacrylamide gels. A 75-bp DNA with no adenine blocks or bulged bases served as a control, and a synthetic CF DNA with a 3-base gap in one strand was also included; 1310 molecules scored. In a separate experiment, four DNAs were prepared from synthetic oligonucleotides (Fig. 1*A*): a DNA with a CTT bulge in the top strand shown in Fig. 1*A*, a DNA with an AAG bulge in the bottom strand, an 85-bp DNA with no deletion in either strand, and an 82-bp DNA with the 3-base deletion in both strands; 1274 molecules scored. The DNAs were prepared for EM as described under "Experimental Procedures." In fields of molecules, the number that were straight or bent by less than 30° were scored against those which appeared to have a central kink of 30° or greater, as judged by eye. Measurements in parentheses were made quantitatively from micrographs in the latter class.

DNA	% molecules straight	% molecules with a bend at the center
PCR-amplified		
Heteroduplexes	58	42 (14)[a]
Homoduplexes	76	24 (3)
75-bp homoduplex	83	17 (2)
DNA with a 3-base gap	67	33 (9)
Synthetic		
CTT-bulged DNA	62	38 (13)
AAG-bulged DNA	61	39 (12)
85-bp DNA	81	19 (3)
82-bp DNA	81	19 (3)

[a] Number in parentheses indicates the percent of the total molecules that contain a kink 40–60% from one end of the DNA and in which the kink measures between 70 and 90°. A kink of 0° represents a perfectly straight molecule.

A 75-bp DNA lacking bulges or oligo(dA) tracts (see below) was analyzed as a control, and it showed a lower fraction with a central kink (Table I) as well as a similar angular distribution to the homoduplex DNA (Fig. 3). In addition, the 3-base gapped DNA showed fewer kinked molecules (Table I) and a broader distribution of kinking angles (Fig. 3) compared with the bulged DNA.

Examination of the DNAs synthesized to contain just the CTT or AAG bulges and the respective homoduplexes revealed a similar distribution of kinked and straight molecules as seen in the PCR-amplified DNA (Table I), and the angular distribution of kinks (not shown) was nearly identical for both.

DISCUSSION

This study provides the first direct visualization of a bulge-induced kink in DNA and confirms earlier conclusions that the slower electrophoretic mobility of bulge-containing DNA results from its bent conformation (6). Inspection of the angular distribution of kinking angles showed that the presence of a single 3-base bulge produced 5–7-fold more molecules containing a central kink of 80 ± 10° than was found in the non-bulged DNAs. Gel electrophoresis revealed that the CTT bulge kinks DNA less than an AAG bulge, although this difference was not detected by EM.

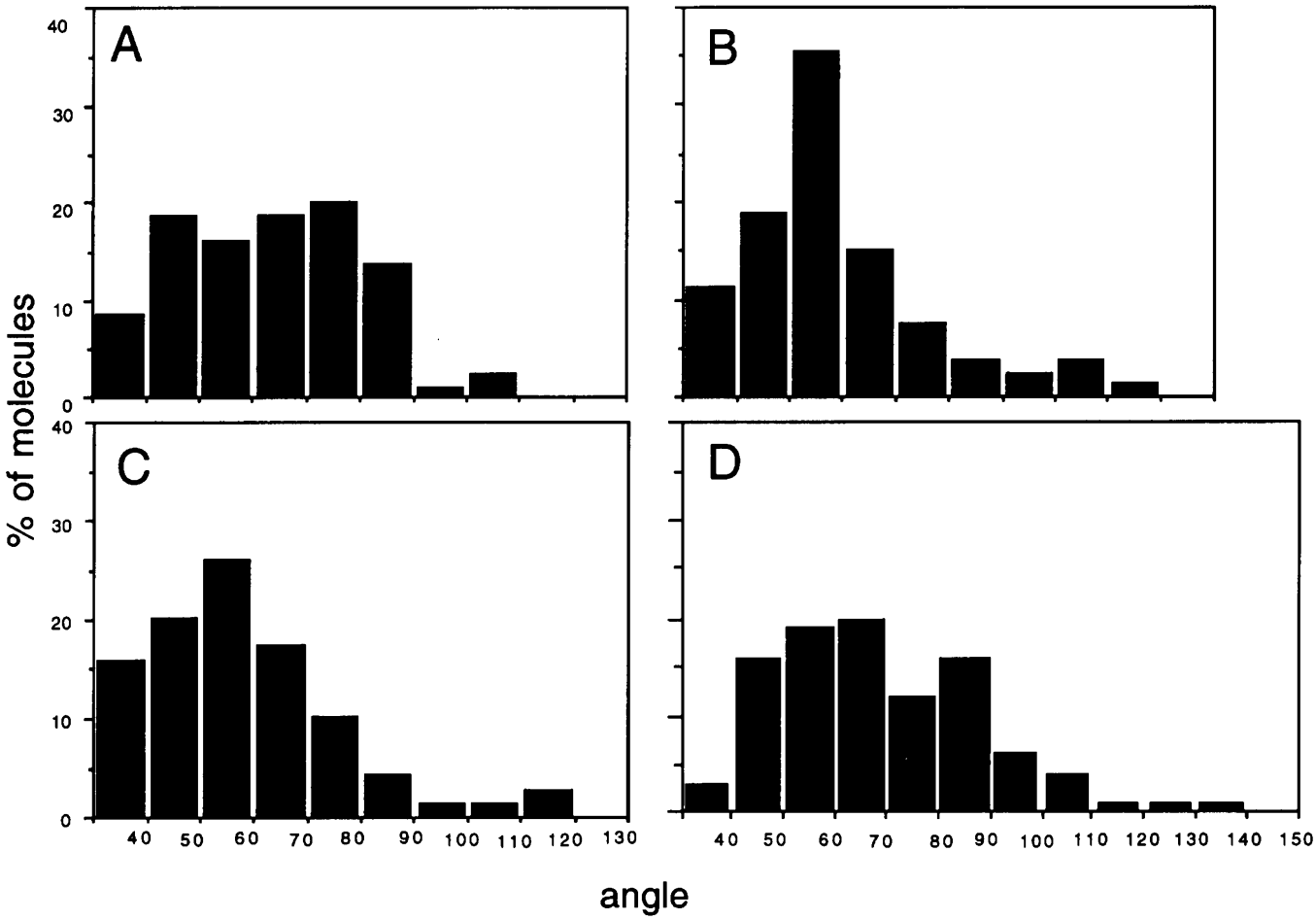

FIG. 3. **Angular distribution of kinking angles.** The CF DNAs from the PCR amplification, a pool of both heteroduplexes (*A*) and both homoduplexes (*B*), together with the 75-bp control DNA (*C*) and the DNA containing a 3-base gap (*D*) were prepared for EM as described under "Experimental Procedures." From the micrographs, the angle of the kink in each kinked DNA was measured as well as its position relative to the DNA ends. For those molecules in which the kink was within 10% of the center, the angle of the kink was plotted. Approximately 100 bent molecules for each sample were measured. A cut-off of angles less than 30° was imposed by eye.

Two indirect estimates of the magnitude of the kink produced by bulged bases have been derived from comparisons with the electrophoretic mobility of oligo(dA) tract-containing DNAs. From the data of Hsieh and Griffith (6), one can estimate that 1 bulged base may kink DNA by 30°, and Rice and Crothers (7) arrived at a similar value. Both of these estimates however assumed that the equilibrium distribution between bent and unbent states for oligo(dA) tract and bulge-containing DNAs are the same, and that is not likely to be the case.

The CF gene contains a tract of 4 adenines, 6 nucleotides 5′ to the beginning of the 3-base deletion (Fig. 1*A*). If this tract were to cause a sequence-directed bend, it would be in the same direction as the bulge-induced bend. Current estimates indicate that in DNAs with multiple phased adenine tracts, a tract of 6 adenines bends DNA by between 11 and 28° (19), but 4 adenines bend DNA substantially less (20) and, in some cases, depending on sequence environment, can cause little or no bending (21). Thus the contribution of this adenine tract is likely to be minimal. It is of interest that adenine tracts can interrupt polymerase movement (22); possibly this nearby adenine tract may have played a role in the generation of the 3-base deletion ΔF_{508} *in vivo*.

Although both the bulged and non-bulged DNAs migrated as sharp bands on the polyacrylamide gels, the distribution of kinking angles measured by EM was broad. This is to be expected for several reasons. Driven by thermal fluctuations, normal non-bulged, non-bent DNA molecules exist in solution in a rapid equilibrium between many different shapes, yet migrate as a single species in gels with a mobility that represents a time-averaged shape. An extreme example of this can be found in the electrophoretic behavior of the highly bent 223-bp kinetoplast DNA from *Crithidia fasciculata* (21, 23) (Fig. 4*A*). On 6% polyacrylamide gels, the 223-bp DNA migrates with an apparent size (relative to non-bent markers) of 850 bp; when closed into a circle with DNA ligase, it appears to be 1350 bp in size, and when stiffened with distamycin, it migrates with an apparent size of 223 bp. As illustrated in Fig. 4*A*, the mobility of 850 bp is an average, reflecting a rapid equilibrium between shapes from straight to perfectly circular. Indeed, electron micrographs of this DNA (16, 23) revealed a full range of such forms in excellent agreement with this analysis. Thus the observation that the bulged DNAs migrate as a single species, yet show a distribution of kinking angles was expected. Here, EM has provided a snapshot of the different forms allowing us to measure the distribution of kinking angles.

The DNAs containing a 3-base gap showed an even broader distribution of kinking angles than the bulged DNA. This is also to be expected since, as illustrated in Fig. 4*B*, a bulged

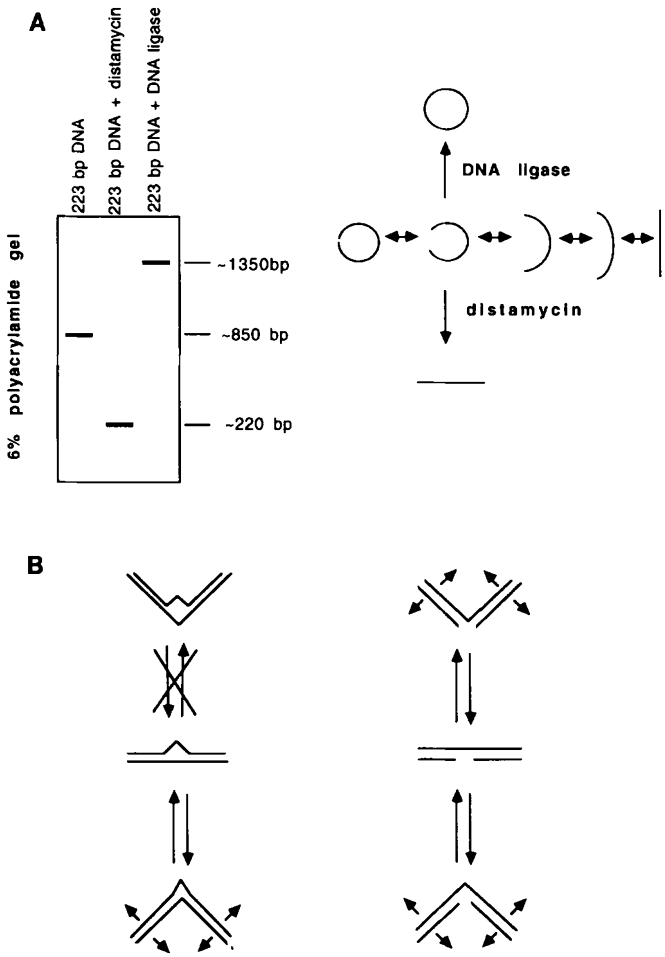

FIG. 4. *A*, schematic illustration of the electrophoretic behavior of the highly bent 223-bp kinetoplast DNA from *C. fasciculata* (16, 23) following ligation or treatment with distamycin. *B*, illustration of the greater constraints on kinking for bulged DNA in contrast to gapped DNA.

DNA may be restricted from bending in certain directions, in particular into the bulge. A gap, however, should produce a flexible joint that would allow bending in all directions. From the micrographs we were unable to determine the direction of kinking of the molecules; nonetheless, our results argue that the kinks produced by the 3-base bulges examined here are less flexible than the 3-base gaps.

Bulged bases produce lesions in DNA, but in RNA they are one of the fundamental elements which contribute to its three-dimensional folding. Extra bases have been shown to kink duplex segments of RNA (24), but little is known about the magnitude of these kinks or their sequence dependence. Here, we have shown that bulge-induced kinks can be visualized by EM. In the future, such EM studies could provide a valuable experimental tool in elucidating the structure of natural RNA.

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